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Role of phospholemman and the 70 kDa inhibitor protein in regulating Na^+/K^+ ATPase activity in pulmonary artery smooth muscle cells under U46619 stimulation

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ABSTRACT

Treatment of bovine pulmonary smooth muscle cells with U46619 inhibited the Na^+/K^+ ATPase activity in two parallel pathways: one of which is mediated via glutathionylation of the pump and the other by augmenting the inhibitory activity of the 70 kDa inhibitor protein of Na^+/K^+ ATPase. Although phospholemman deglutathionylates the pump leading to its activation, the inhibitor is responsible for irreversible inhibition of Na^+/K^+ ATPase in an isoform specific manner during treatment of the cells with U46619.

1. Introduction

The Na^+/K^+ ATPase (NKA) is a ubiquitous plasma membrane bound enzyme, generates transmembrane concentration gradients for Na^+ and K^+ that are essential for the cells to function. The enzyme is composed of two essentially non-covalently linked subunits, viz., the α subunit, which is responsible for catalytic activity of the pump; and the β subunit, which is necessary for the stabilization and maturation as well as function of the pump [1–3].

In recent time, the FXYD proteins, having the signature FXYD motif in the N-terminal, are found to be novel regulators of NKA [4,5]. At present, the FXYD family has at least 12 members. The γ-subunit of NKA is the only member in the FXYD family that has two alternative splice variants (FXYD2a and FXYD2b) [5]. As a family, FXYD proteins are found predominantly in tissues like kidney, colon, pancreas, etc. (that are involved in solute and fluid transport) and also in electrically excitable tissues (heart, skeletal muscle, etc.) [5]. Different protein kinase (s) can phosphorylate the FXYD proteins due to presence of potential phosphorylation sites onto their cytoplasmic tail [6–10]. An interesting finding from our laboratory shows that PKA and PKC phosphorylate PLM depending on its association with different isoforms of NKA [11].

Oxidative modification can regulate protein functions. It may provide an alternative to phosphorylation of the NKA or associated FXYD because the pump's subunits contain residues that can be oxidized (e.g. the sulfhydryl group) [12] and it has been shown that NKA in skeletal muscle is redox sensitive [13]. Bibert et al. [12] recently showed that angiotensin II induces PKC activity and that leads to ROS generation, which subsequently causes glutathionylation of the β subunit and hence inhibits NKA activity. On the other hand, it has been shown that oxidant stress can also activates NKA activity. A bolus of H_2O_2 activates NKA in cardiac myocytes [14]. The report from Bibert et al. [12] also seems interesting in that context. They showed that although peroxynitrite inhibits the pump by gulatathionylation of its β subunit, yet PLM relieved this inhibition by facilitating deglutathionylation of the pump.

Inhibition of NKA is known to be dependent upon its conformation. Ouabain has been shown to inhibit NKA activity by binding to the E2 state of the enzyme [15]. Recently, we have reported a 70 kDa inhibitor protein of NKA in bovine pulmonary artery smooth muscle (BPASM) that inhibits the pump upon binding to its E1 site. We have also observed that the inhibitor showed higher affinity towards the α_2 isoform of NKA than the α_1 isoform [16].

In this present communication, we examined in BPASM cells (BPASMCs), the regulation of NKA by the TxA_2 mimetic U46619

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and seek to determine whether this is mediated by oxidative modification of the pump as we have previously reported that U46619 increases O_2^- generation via stimulation of NADPH oxidase activity in BPASMCs [17]. Recently, we have reported the presence of PLM in smooth muscle cells [11] and hence we investigated its involvement in this scenario. Huang et al. [18] showed that different isoforms of NKA are differentially inhibited by oxidants. Since BPASMCs contain both the α_1 and α_2 isoforms of NKA [11], therefore, one of our aims was to investigate the isoform specific regulation of NKA by oxidants and to explore the underlying mechanisms. In view of our recent report about the presence of a 70 kDa inhibitor of NKA [16], we also determined its role in regulating the enzyme activity upon stimulation of the cells with U46619.

2. Materials and methods

2.1. Materials

All the chemicals were the products of Sigma Chemical Co. (USA) unless otherwise stated. NKA β_1-subunit and α-subunit (anti-LEAVE) specific polyclonal antibody was kindly provided by Prof. Pablo Martin Vassalo, Department of Biochemistry and Molecular Biology, University of La Lagune, La Lagune (Tenerife, Spain) and by Prof. Tomas Pressley, Department of Physiology, Texas Tech University Health Sciences Center [19]. Custom made anti-PLM antibody was prepared according to Dey et al. [11].

2.2. Cell culture

BPASMCs were studied between passages 6 and 12. Cells were maintained according to our previously described protocol [11].

2.3. Generation of antibody against the 70 kDa inhibitor protein of NKA

Antibodies to purified inhibitor were raised in rabbit [20]. The rabbit was injected subcutaneously as follows with the inhibitor emulsified with Freund's reagent (Freund's complete adjuvant): 120 mg of inhibitor on day 1 and 100 mg on day 7. This was followed by intramuscular injection of 20 mg of the inhibitor on days 28 and 35. Serum was collected on day 45 for affinity purification.

2.4. Reconstitution of NKA and PLM into liposomes

The purified fraction containing both the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozymes of NKA was reconstituted with or without PLM into the liposomes which were prepared using the lipid DOPC by following the procedure of Dey et al. [11]. Two other liposomal systems were prepared which were reconstituted with PLM, 70 kDa inhibitor and either with $\alpha_1\beta_1$ or with $\alpha_2\beta_1$ isozymes of NKA.

2.5. Na^+ efflux through the NKA

NKA flux in the reconstituted liposomes was determined as the rate of pump-mediated $[Na^+]_i$ decline by following the procedure of Dey et al. [11].

2.6. Glutathionylation of NKA

To detect *S*-glutathionylation of the pump subunits, cells were loaded with biotinylated GSH. After lysis, the membrane fraction was isolated and the biotin-tagged glutathionylated subfraction was precipitated using streptavidin–sepharose beads [21] and immunoblotted for α and β_1 NKA subunits. In separate experiments the β_1 subunit immunoprecipitated from the cell membrane fraction was immunoblotted with an antibody against glutathionylated protein (anti-GSH antibody, Invitrogen). When indicated, the enzyme was exposed to 10 nM U46619 before biotin-GSH ester was added at different time points. In vitro glutathionylation was performed according to Passarelli et al. [22] with 1 mM GSH and 1 mM H_2O_2 and by using 0.05% Triton X 100 to permeate the liposomes.

To detect glutathionylation of the pump in its different conformations, we used different buffer compositions to stabilize them. For E1ATP: 0.1 mM EDTA, 25 mM histidine, 2 mM Na_2ATP (pH 7.1); for E2: 0.1 mM EDTA, 25 mM histidine; for E1Na_3: 100 mM NaCl, 4 mM $MgCl_2$, 20 mM histidine (pH 7.35).

2.7. Expression of 70 kDa protein

The level of 70 kDa inhibitor protein was measured in the cytosol of BPASMCs by western blot study as previously described [11] with polyclonal antibody against the said protein raised in rabbit.

2.8. Immunofluorescence study

Cells were fixed for the immunofluorescence study according to Santos et al. [23]. Antibody raised against the inhibitor protein was used as primary antibody. FITC labeled anti-IgG was used as secondary antibody.

2.9. Measurement of ATPase activity

NKA activity was measured as previously described [11] with or without U46619 treatment at different time points.

2.10. Statistical analysis

Data were analyzed by unpaired *t* test and analysis of variance, followed by the test of least significant differences for comparison within and between the groups [24] (Daniel 1978). $P < 0.05$ was considered significant.

3. Results

3.1. Glutathionylation of NKA

We examined the identification (Fig. 1A and B; lane 1) and glutathionylation pattern (Fig. 1A and B; lanes 2 and 3) of different subunits of NKA after treatment of BPASMCs with U46619. Cells loaded with biotin-GSH were exposed to solutions containing 10 nM U46619 for 10 min. U46619 increased β_1 subunit glutathionylation detected by immunoblotting the glutathionylated protein (GSS-protein) precipitated by streptavidin as shown in Fig. 1B. The α_1 subunit was not detected in the GSS-protein sub fraction after oxidant treatment (Fig. 1A). To gain further support for glutathionylation, we immunoprecipitated the β_1 subunit and immunoblotted the precipitate with antibody against GSS-protein. The reverse immunoprecipitation experiment was also performed as shown in Fig. 1A and B (Lanes 4 and 5).

Cells treated with U46619 showed a large signal for glutathionylation when the buffering condition stabilizes the E1 conformation (Fig. 1C, lane 1 for E1ATP and lane 4 for E1Na_3) whereas the signal was much smaller in a solution that stabilizes the E2 conformation (Fig. 1C, lane 2) with the biotin-GSH technique. For further confirmation, cells were treated with 100 μM ouabain and then with U46619. Glutathionylation was measured after 10 min. The signals for glutathionylation were also low in this case

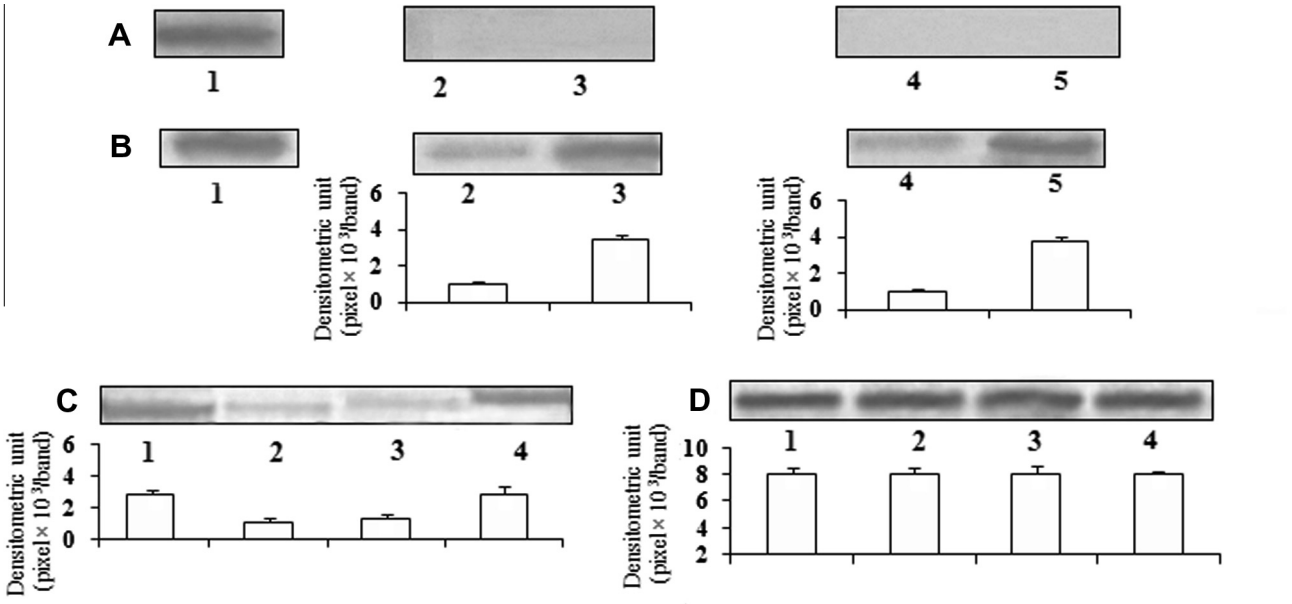

Fig. 1. (A and B) Glutathionylation of α and β subunits of NKA in BPASMCs. Lane 1: Identification of α and β_1 subunit with anti-LEAVE and β_1 subunit specific antibody in the cell membrane fraction (50 μg of total protein). Lanes 2 and 3: Determination of glutathionylation pattern (by biotin-GSH technique) at basal and after U46619 treatment conditions. It was found that only the β_1 subunit is susceptible for glutathionylation after treatment with U46619. A similar result was found with GSH antibody technique (lanes 4–5). (C) Glutathionylation pattern obtained when the pump was allowed to be stabilized in the E1 conformation (lane 1: E1ATP, lane 4: $E1Na_3$ conformation) and in the E2 conformation (lane 2). Lane 3: glutathionylation of the pump after treatment with ouabain. (D) Annexin II is used for loading control.

indicating that glutathionylation favor the E1 conformation as ouabain stabilized the E2 conformation of the pump (Fig. 1C, lane 3).

3.2. Effect of U46619 on NKA activity and its glutathionylation level: role of PLM and inhibitor protein

From western blot profile it was found that U46619 treatment increases the glutathionylation signal for both PLM and NKA β_1 subunit (Fig. 2A and B, lanes 2 and 3). However, after 15–20 min, glutathionylation of NKA goes on decreasing (Fig. 2B, lane 4) although the expression levels of both the proteins remain same (Fig. 2C and D). Similar results were also found by Bibert et al. [12] in case of cardiac myocytes where they showed that FXYD proteins facilitated the deglutathionylation of NKA and hence a low glutathionylation signal was obtained. However, to ascertain the role of PLM in this scenario, we performed studies with reconstituted liposomes. We used two different kinds of liposomal systems for this purpose: one of which was reconstituted with $\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozymes of NKA and the other reconstituted with both the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozymes of NKA and PLM. No change in the glutathionylation signal was observed in case of the liposomes reconstituted with $\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozymes of NKA (Fig. 2E); while in case of the liposomes reconstituted with both NKA ($\alpha_1\beta_1$ and $\alpha_2\beta_1$ isoforms) and PLM, signal for β_1-glutathionylation decreases after 20 min (Fig. 2F, lane 3). These results indicated that PLM facilitated deglutathionylation of NKA. We next determined the role of 'glutathionylation–deglutathionylation' status of the pump on its ATPase activity in the reconstituted liposomes. With decreasing level of glutathionylation, the ATPase activity of the pump increases after 20 min of U46619 treatment (Fig. 3A vs. B). Interestingly, a different observation was found with the pump activity in BPASMCs. Cells treated with U46619 for 10 min decreases the ATPase activity with increase in the glutathionylation level of the pump (Fig. 3C, lane 2). But, deglutathionylation of the pump by PLM after 20 min did not restore the basal pump activity (Fig. 3C, lane 3) as observed in reconstituted liposomes, indicating an irreversible inhibition on the ATPase activity during oxidative stress. We found an increase in inhibitory activity of the 70 kDa protein, which we have recently identified against the pump during U46619 stimulation. To ascertain the role of the inhibitor protein on the pump activity after U46619 treatment, cells were first permeabilised with 0.01% (v/v) Triton X-100 in PBS for 15 min at room temperature. This treatment restores the normal morphological integrity of the cells as suggested by Santos et al. [23]. Next the cells were loaded with polyclonal antibody against the inhibitor followed by treatment with U46619 (10 nM) and after 20 min the pump activity was measured. Blocking the inhibitor activity of the 70 kDa protein with its antibody showed that the pump activity was greater compared to that observed without the antibody pretreatment (Fig. 3C, lane 4) and after ~40 min, it almost reaches the basal value (Fig. 3C, lane 5). However, from western blot profile (Fig. 4A), it was observed that the expression level of the inhibitor protein was not altered during oxidative stress caused by U46619 (Fig. 4A). The binding of the antibody to the inhibitor protein was confirmed by immunofluorescence study (Fig. 4B).

To investigate the role of the 70 kDa inhibitor on different isoforms of NKA under oxidative stress, we performed in vitro glutathionylation and measured the ATPase activity in the liposomes reconstituted with different isozymes of NKA (either $\alpha_1\beta_1$ or $\alpha_2\beta_1$) in conjugation with PLM and the 70 kDa inhibitor protein. Measurement of ATPase activity (Fig. 3D vs. E) clearly showed that under oxidative stress the inhibitor inhibited the $\alpha_2\beta_1$ isozyme more than the $\alpha_1\beta_1$ isozyme of NKA.

3.3. Role of NADPH oxidase

It was observed that only the β subunit of the pump is susceptible to glutathionylation when the cells were treated with U46619. Moreover, the U46619 induced glutathionylation was abolished by pre incubating the cells with SQ29584 (TxA_2 receptor antagonist: 1 μM), SOD (O_2^- scavenger: 200 μM) and ebselen ($ONOO^-$ scavenger: 1 μM) for 5 min (Fig. 5). These results clearly indicated that U46619 glutathionylate the pump in NADPH oxidase dependent mechanism.

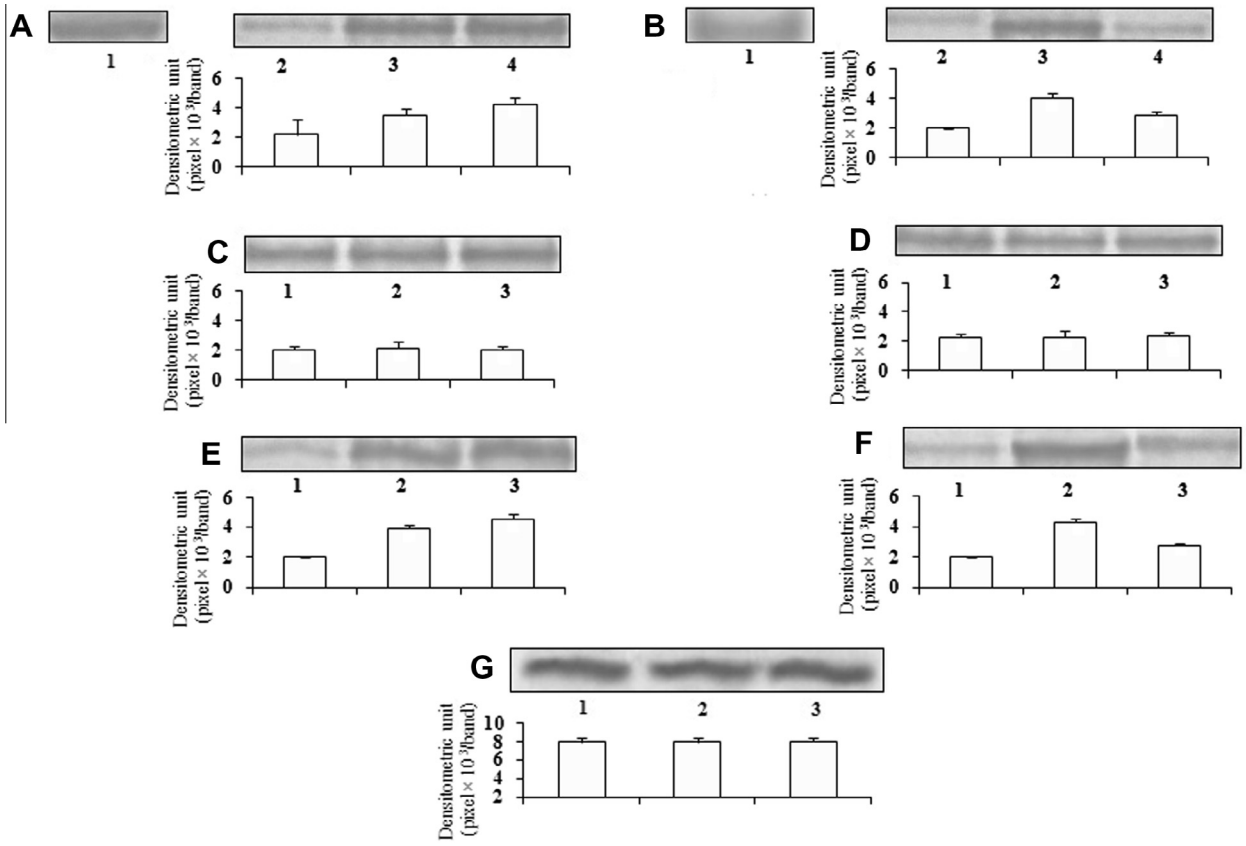

Fig. 2. Identification of glutaredoxin1 and time dependent glutathionylation (with anti-GSH antibody technique) of PLM as well as NKA (50 μg of total protein was loaded onto the wells). (A) Lane 1: Identification of PLM in the cell membrane of BPASMCs; lanes 2–4: glutathionylation pattern of PLM at basal, after 10 min and after 20 min of U46619 treatment, respectively. (B) Lane 1: Identification of GRX1 in the cell membrane of BPASMCs; lanes 2–4: glutathionylation pattern of NKA at basal, after 10 min and after 20 min of U46619 treatment, respectively. (C and D) Represent the protein expression profile of PLM and NKA. Glutathionylation of NKA ($\alpha_1\beta_1$ and $\alpha_2\beta_1$ isoforms) alone (E) and in presence of PLM (F) in reconstituted liposomes; lane 1: glutathionylation under basal condition; lanes 2 and 3: glutathionylation after 10 and 20 min, respectively. (G) Annexin II is used for loading control.

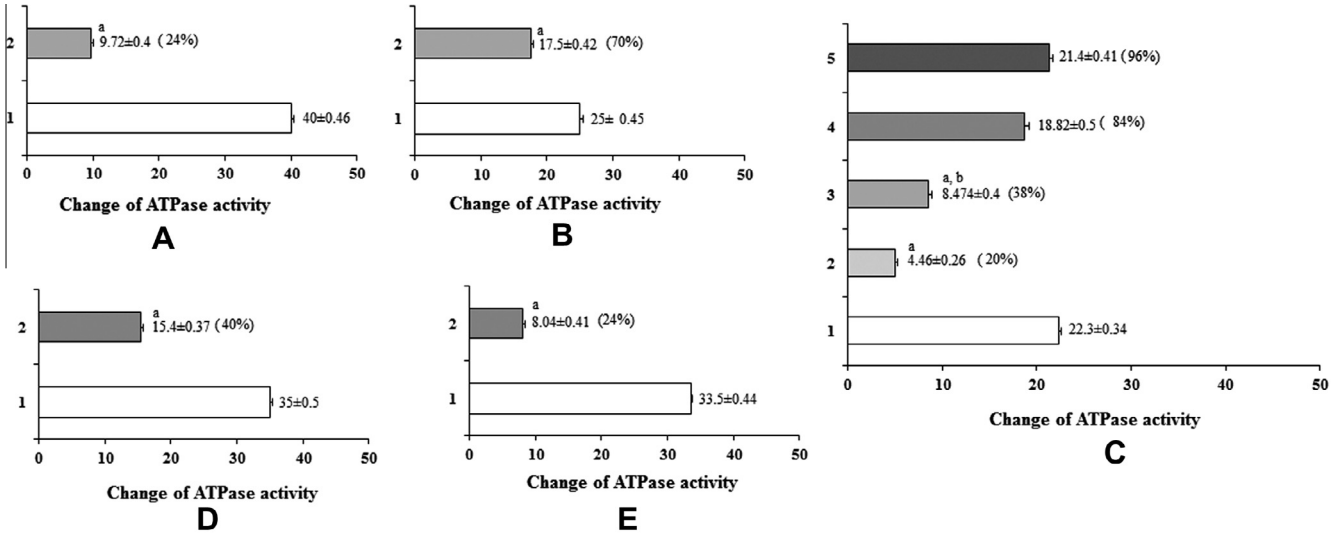

Fig. 3. Change of NKA activity in reconstituted liposomes and in BPASMCs. (A and B) Proteoliposomes reconstituted with NKA ($\alpha_1\beta_1$ and $\alpha_2\beta_1$ isoforms) alone and in conjugation with PLM: lanes 1 and 2, ATPase activity at basal and after 20 min of U46619 treatment, respectively. (C) ATPase activity in BPASMCs: lanes 1–3, ATPase activity at basal condition, after 10 min and after 20 min of U46619 treatment, respectively; lanes 4 and 5, ATPase activity after 20 min and 40 min of U46619 treatment in presence of antibody against the 70 kDa inhibitor of NKA. (D and E) Proteoliposomes reconstituted with either $\alpha_1\beta_1$ or $\alpha_2\beta_1$ isozyme of NKA along with PLM and 70 kDa inhibitor, respectively: lanes 1 and 2, ATPase activity at basal and after 20 min of U46619 treatment. Results are mean ± SE ($n = 4$). [a]$P < 0.05$ compared to basal condition; [b]$P < 0.05$ compared to lane 4.

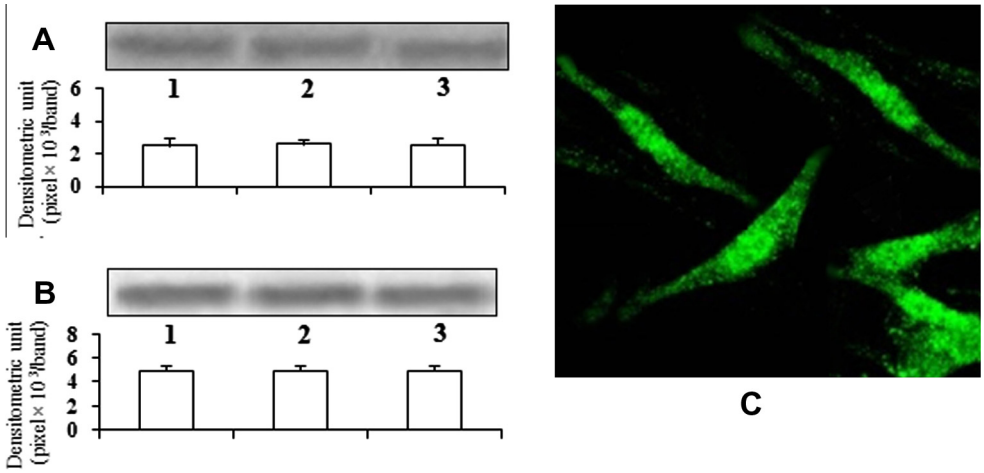

Fig. 4. Expression of 70 kDa inhibitor protein in the cytosol of BPASMCs (30 μg of total protein was loaded onto the wells). (A) Western blot study: lanes 1–3, untreated cells, after 10 min and after 20 min of treatment with U46619, respectively. (B) β-Actin is used as a loading control. (C) Immunofluorescence study using FITC conjugated anti-70 kDa inhibitor antibody.

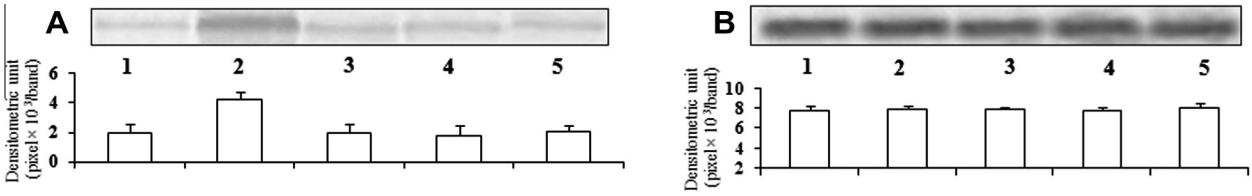

Fig. 5. Role of NADPH oxidase on the glutathionylation level of Na^+/K^+ ATPase (50 μg of total protein was loaded onto the wells). (A) Lane 1: Basal glutathionylation. Lane 2: Glutathionylation after 10 min of U46619 treatment. Lanes 3–5: Glutathionylation pattern after preincubating the cells with SQ 29548, SOD and with ebselen, respectively. (B) β-Actin is used as a loading control.

4. Discussion

In this communication, we examined the regulation of NKA during stimulation of BPASMCs with the TxA_2 mimetic, U46619. We also examined the role of PLM in this scenario. Interestingly, we observed an important role of the 70 kDa inhibitor protein towards the irreversible inhibition of NKA under U46619 mediated oxidative stress.

The origin of ROS in the vessel wall is currently of immense interest, because accumulating evidence suggests that these molecules participate in the pathogenesis of vascular diseases [25]. We have recently demonstrated that NADPH oxidase is a major source of ROS in vascular smooth muscle cells [17]. White et al. [26] demonstrated that angiotensin II by producing $ONOO^-$ inhibits the NKA activity due to glutathionylation of β subunit of the pump in a NADPH oxidase dependent manner.

We found that only the β subunit (specifically the β_1 subunit) of the pump was susceptible to glutathionylation when the cells were treated with U46619 and it was interesting to note that this glutathionylation of NKA was conformation dependent. Glutathionylation inhibits the pump by stabilizing its E1 conformation. Figtree et al. [27] showed that glutathionylation of NKA is reversible in nature where glutaredoxin1 (GRX1) facilitated deglutathionylation of the pump. In BPASMCs, we determined the presence of GRX1 (Fig. 2B, lane 1), but the expected glutathionylation signal was quite different after its inhibition. Bibert et al. [12] showed that recombinant FXYD3 facilitated the deglutathionylation of NKA in *Xenopus oocytes*. We found that glutathionylation of PLM was induced from baseline by exposing the cells to U46619. We also found that after 20 min of U46619 treatment, the signal for glutathionylation of NKA decreases considerably. We propose that there might be two possibilities to explain these results: either PLM facilitates the deglutathionylation of NKA or GRX1 might have a contribution. Again if GRX1 was involved, then its inhibition might prevent the loss of glutathionylation signal of NKA. But, we did not find any discernible contribution from GRX1 during the oxidative stress in the cells (Fig. 2B, lane 4), which clearly suggest the possibility that GRX1 might be inhibited under this condition. Inhibition of GRX1 under oxidative stress has also been suggested by Heshemy et al. [28]. To ascertain the role of PLM in this scenario, we performed studies with liposomes reconstituted with NKA ($\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozymes) and PLM. No change in the glutathionylation signal was observed after 30 min of U46619 treatment in case of the liposomes reconstituted with $\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozymes of NKA. However, results obtained from the liposomes reconstituted with both PLM and NKA ($\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozyme) were different. It clearly suggests that PLM facilitated deglutathionylation of NKA β_1 subunit. A similar result was also found from ATPase activity measurement in reconstituted liposomes.

Although PLM deglutathionylates the β_1 subunit of NKA, yet measurement of ATPase activity in the cells did not corroborate with the results obtained from liposomes reconstituted with either $\alpha_1\beta_1$ or $\alpha_2\beta_1$ isozymes NKA and PLM. Bibert et al. [12] showed that the ATPase activity was proportional with the level of glutathionylation of the pump, but we have not found such proportionality after treatment of the cells with U46619. It is also well established that oxidant stress during ischemic condition may cause accumulation of substances that inhibit membrane transporter such as NKA. Fuller et al. [29] showed that an unstable cardiac and brain-specific inhibitor of the NKA, whose production is linked to oxidant stress, accumulates intracellularly during ischemia. Here in case of BPASMCs, we found an increase in the activity of 70 kDa inhibitor protein towards NKA after 20 min of U46619

treatment without any change in the expression level of the protein.

The mechanism of U46619 induced increase in the inhibitor activity of the 70 kDa protein in the cells is currently unknown. Conceivably, in BPASMCs, two mechanisms were simultaneous operating in inhibiting NKA by producing $ONOO^-$ in NADPH oxidase dependent manner under U46619 treatment to the cells. One operated through glutathionylation of the pump, while the other appears to occur via the inhibitor protein. These two pathways were linked with a common mechanism. Glutathionylation inhibit the pump by stabilizing its E1 conformation, and the inhibitor binds with the pump in the same conformation. Apparently, this may explain the irreversible inhibition of NKA activity under oxidant stress by U46619 even in presence of PLM. This result although contradicts the work of Figtree et al. [26], but oxidant mediated irreversible inhibition of the pump was also reported by Huang et al. [30]. Interestingly, functional inhibition of the 70 kDa inhibitor with its polyclonal antibody restores the basal ATPase activity. Deactivation of the inhibitor protein due to binding of the antibody was confirmed by immunofluorescence study. Huang et al. [18] showed that the α_2 and α_3 isoforms are more susceptible than the α_1 isoform toward oxidants. In BPASMCs, oxidant induce the inhibitory activity of the 70 kDa inhibitor protein, which have different affinities toward different isoforms of NKA (α_2 isoform is more susceptible than α_1) resulting in the differential regulation of the NKA isoforms.

5. Conclusion

Our present study suggests that in addition to the inhibition of NKA through glutathionylation, a marked increase in the inhibition of the enzyme occurs via activation of the 70 kDa inhibitor protein under U46619 stimulation to the cells. We also suggest that the presence of this inhibitor is solely responsible for isoform specific irreversible inhibition of NKA by oxidants during U46619 stimulation of the cells.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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